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Marrow-Derived Stromal Cells Express Genes Encoding a Broad Spectrum of Arteriogenic Cytokines and Promote In Vitro and In Vivo Arteriogenesis Through Paracrine Mechanisms

T. Kinnaird, E. Stabile, M.S. Burnett, C.W. Lee, S. Barr, S. Fuchs, S.E. Epstein

Abstract—We recently demonstrated that marrow stromal cells (MSCs) augment collateral remodeling through release of several cytokines such as VEGF and bFGF rather than via cell incorporation into new or remodeling vessels. The present study was designed to characterize the full spectrum of cytokine genes expressed by MSCs and to further examine the role of paracrine mechanisms that underpin their therapeutic potential. Normal human MSCs were cultured under normoxic or hypoxic conditions for 72 hours. The gene expression profile of the cells was determined using Affymetrix GeneChips representing 12 000 genes. A wide array of arteriogenic cytokine genes were expressed at baseline, and several were induced >1.5-fold by hypoxic stress. The gene array data were confirmed using ELISA assays and immunoblotting of the MSC conditioned media (MSC^{CM}). MSC^{CM} promoted in vitro proliferation and migration of endothelial cells in a dose-dependent manner; anti-VEGF and anti-FGF antibodies only partially attenuated these effects. Similarly, MSC^{CM} promoted smooth muscle cell proliferation and migration in a dose-dependent manner. Using a murine hindlimb ischemia model, murine MSC^{CM} enhanced collateral flow recovery and remodeling, improved limb function, reduced the incidence of autoamputation, and attenuated muscle atrophy compared with control media. These data indicate that paracrine signaling is an important mediator of bone marrow cell therapy in tissue ischemia, and that cell incorporation into vessels is not a prerequisite for their effects. (*Circ Res.* **2004;94:678-685.**)

Key Words: marrow stromal cells ■ arteriogenesis ■ bone marrow cells ■ cytokines

An important compensatory response to atherosclerotic obstructive arterial disease is collateral development, a complex process requiring that multiple genes coordinately express their products in an appropriate time-dependent manner.[1,2] However, the natural capacity of collaterals to remodel and enlarge to compensate for the reduced flow that occurs after occlusion of a major artery is rarely sufficient to restore maximal flow capacity to levels required under various stress-conditions.

Although several protein and gene-based strategies have succeeded in enhancing collateral development in animal models of ischemia, clinical studies thus far have been disappointing.[3–5] Given that the natural response to tissue ischemia is such a complex process, the delivery of a single growth factor may be too simple an approach. Thus, a great deal of interest has arisen in the potential of cell-based strategies in augmenting collateral responses, and several groups have demonstrated incorporation of various bone marrow–derived cells into new or remodeling vessels.[6,7]

However, the actual magnitude of incorporation of bone marrow–derived cells into vascular structures varies substantially between studies. Although some studies report over 50% of capillaries containing transplanted cells, other studies have reported only occasional positive vessels despite impressive improvement in perfusion.[8–10] Taken together, these data suggest that other mechanisms apart from cell incorporation may contribute to collateral remodeling observed after bone marrow–derived cell therapy in various models of ischemia. Furthermore, we recently demonstrated that marrow stromal cells (MSCs) augment collateral remodeling through release of several cytokines such as VEGF and bFGF rather than via cell incorporation into new or remodeling vessels.[11] Therefore, the purpose of the present study is to characterize the full spectrum of cytokine genes expressed by MSCs and to further examine the role of paracrine mechanisms that underpin the biological effects of MSC therapy for tissue ischemia.

Materials and Methods

Human Cell Lines

Normal human MSCs (derived from a single 19-year-old healthy donor) were purchased from Clonetics (Walkersville, Md) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (DM-10). These cells were previously demonstrated to

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be negative for CD34 and CD45 surface markers, and positive for CD44, CD105, and CD166. Passages 3 to 5 were used for in vitro experiments. Human umbilical vein endothelial cells (ECs) were purchased from American Type Culture Collection (ATCC, Manassas, Va) and cultured in endothelial growth media-2 (EGM-2; Clonetics). Human aortic smooth muscle cells (SMCs) were purchased from ATCC and cultured in Medium-199 supplemented with 10% fetal bovine medium and 1% penicillin-streptomycin (M-10). Passages 3 to 8 were used for in vitro experiments. Cells were cultured in 20% O_2 and 5% CO_2 during normoxia experiments, and in 1% O_2 and 5% CO_2 using a hypoxia chamber for hypoxia experiments.

RNA Preparation

Total RNA was extracted from normoxia and hypoxia exposed (72 hours) human MSCs (2 plates per analysis) using TRIzol Reagent (Invitrogen) according to the manufacture's instructions. RNA was cleaned using a RNeasy mini kit (Qiagen) and stored at −80°C.

Microarray Analysis

Double-stranded cDNA was synthesized from 8 μg of total RNA. For the first cDNA strand synthesis, oligo(dT) primers were annealed to the RNA and extension by reverse transcriptase was performed in the presence of deoxyoligonucleotides. The second strand was synthesized using DNA polymerase I and purified using a phase lock gels-phenol/chloroform extraction, followed by ethanol precipitation. In vitro transcription, using double-stranded cDNA as a template in the presence of biotin-labeled ribonucleotides, was performed by using an Enzo in vitro transcription kit (Enzo Diagnostics). Biotin-labeled cRNA was purified, fragmented, and hybridized to Affymetrix Human Genome U133A GeneChips (Affymetrix, Santa Clara, Calif). Hybridization, washing, antibody amplification, staining, and scanning of probe arrays were performed according to the Affymetrix Technical manual. Scanned raw data were processed with Affymetrix GeneChip v 5.1 software. A hypoxia fold induction of >1.5 was considered significant.

Human Conditioned Media Collection, Preparation, and Analysis

For ELISA, human MSC-conditioned media ($hMSC^{CM}$) was collected after 24 hours of culture, centrifuged at 2000 rpm for 10 minutes, and passed through a 0.3 μm filter. The concentration of MSC^{CM} cytokines was measured using sandwich ELISA kits (VEGF, bFGF, MCP-1, and PlGF; R&D systems). After media collection, cells were lysed and total protein counted with the MicroBCA protein assay (Pierce). ELISA values were corrected for total cell protein. EC-conditioned media and DM-10 were also assayed. For cell proliferation and migration assays, $hMSC^{CM}$ was collected for 72 hours and filtered as above. Dilutions were prepared with DM-10 as appropriate.

For immunoblotting, $hMSC^{CM}$ was collected and prepared as above. Forty micrograms of protein were separated using SDS-PAGE gels (10%) and blotted onto nitrocellulose (Invitrogen). After blocking, blots were incubated with primary antibody to PDGF-β (1:1000, Santa Cruz), angiopoetin-1 (1:1500, Santa Cruz), metalloproteinase-9 (1:500, Santa Cruz), or plasminogen activator (1:1000, Santa Cruz). Membranes were developed with an enhanced chemiluminescence kit (Pierce).

Cell Proliferation Assay

ECs or SMCs (1×10^4/well) were plated in 24-well plates in DMEM with 0.1% fetal calf serum for 24 hours to arrest mitosis. For EC proliferation, the media was replaced with varying dilutions of $hMSC^{CM}$, EGM-2 (positive control), recombinant VEGF 4 ng/mL (positive control, R&D Systems), DM-10 (normal control), or boiled $hMSC^{CM}$ (negative control). Further DM-10 samples were supplemented with recombinant VEGF to concentrations coinciding with the concentration of VEGF present in the conditioned medium. To examine the role of cytokines in isolation, 10 μg/mL anti-VEGF antibody (Sigma), 5 μg/mL anti-FGF antibody (Sigma), or 5 μg/mL anti-PDGF antibody (Sigma) was added to $hMSC^{CM}$ dilutions in additional wells. Further DM-10 samples were also supplemented with recombinant VEGF to concentrations found in the relevant dilutions of $hMSC^{CM}$. For SMC proliferation, the media was replaced with varying dilutions of MSC^{CM}, PDGF (10 ng/mL, positive control, Clonetics), or DM-10 (normal control). Cultures were continued for 72 hours, after which the cells were recovered and counted using a Coulter counter. Data are reported as the mean percentage change in proliferation when compared with control media (DM-10).

Cell Migration Assay

EC and SMC migration assays were performed using Transwell culture chambers (Costar, Corning). Cells were suspended in DMEM supplemented with 2% serum and placed in the top chamber (4×10^5/well). For EC migration, DM-10 (normal control), $hMSC^{CM}$, boiled $hMSC^{CM}$, and VEGF (4 ng/mL, positive control) were added to the lower chamber. For SMC migration, DM-10, $hMSC^{CM}$, boiled $hMSC^{CM}$, and PDGF (10 ng/mL, positive control) were added to the lower chamber. Cells were incubated overnight, and the top layer of the membrane scraped gently to remove any cells. Cells on the lower surface of the membrane were stained using Hema-3 staining kit (Biochemical Sciences). Six random fields per membrane were counted. Data are reported as the mean percentage or fold change in proliferation when compared with control media (DM-10).

Murine MSC Preparation and Culture

Murine bone marrow was harvested by flushing the tibiae and femurs of Balb/C mice (two mice per culture; Jackson Laboratories, Bar Harbor, Maine) with DM-10. The pooled marrow was dispersed, plated in DM-10, and cultured for 72 hours. Nonadherent cells were washed off and adherent cells expanded until confluent (≈7 to 10 days). FACS analysis of up to passage 6 demonstrated persistence of lymphohematopoietic cells ($CD34^+$ or $CD45^+$ or both). Therefore, MSCs were purified from the heterogeneous cultured cells. The $CD34^-/CD45^-$ fraction was isolated by labeling with FITC-conjugated anti-CD34 antibody (Pharmingen) followed by simultaneous incubation with a cocktail of anti-FITC and anti-CD45 magnetic beads (Miltenyi Biotech). Cells were passed through a magnetic column, the double-negative fraction collected, and replated. Repeat FACS analysis was performed and demonstrated that cells did not express CD31, CD34, CD45, and CD117, and expressed high levels of CD44, CD90, and CD105 (data not shown) typical of marrow-derived stromal cells, and in keeping with previous published data.[12] For in vivo experiments, murine MSC^{CM} ($mMSC^{CM}$) was collected after 72 hours and then concentrated 2-fold using Microcon YM-10 centrifugal filters (Amicon). As a control, DM-10 was filtered and concentrated in a likewise fashion.

Animal Surgery and Murine MSC^{CM} injection

All animal interventions were approved by the Animal Care and Use Committee of the MedStar Research Institute. Under narcosis, 12-week-old Balb/C mice (n=5 per group) were subjected to operative intervention to create unilateral hindlimb ischemia. The right femoral artery was exposed in the midthigh, dissected from the femoral vein and nerve, and then ligated just proximal to the popliteal bifurcation. In preliminary studies, MSC^{CM} injection immediately after femoral ligation failed to improve flow recovery. Therefore, in the present study, $mMSC^{CM}$ injection was delayed by 24 hours to allow the mice to partially recover from the surgical insult. A total of 50 μL of $mMSC^{CM}$ or DM-10 was injected into the adductor muscle at four sites adjacent to and within 1 mm of the ligation site. The injections were repeated at 48 hours and 72 hours.

Perfusion Imaging

Laser doppler perfusion imaging (LDPI) (Moor Instruments) was utilized to record serial blood flow measurements. For consistent measurements, imaging was performed after limb hair was removed, and after mice had been placed on a heating plate at 37°C to minimize temperature variation. Calculated perfusion is expressed as a ratio of the ischemic to normal limb.[13] Previous data have

Marrow-Derived Stromal Cells Proangiogenic/Proarteriogenic Gene Expression

Cytokine	Angiogenic/Arteriogenic Function	Fold Induction With Hypoxia
Angiopoetin-1	EC migration, vessel stabilization	...
Fibroblast growth factor-2	EC and SMC proliferation and migration	1.62
Fibroblast growth factor-7	EC proliferation and stabilization	1.82
Hepatoma growth factor	SMC proliferation	...
Interleukin-1	VEGF induction	1.91
Interleukin-6	VEGF induction	2.26
Metalloproteinase-1	Loosens matrix, tubule formation	...
Metalloproteinase-2	Loosens matrix, tubule formation	...
Metalloproteinase-9	Loosens matrix	...
MCP-1	Monocyte migration	...
M-CSF	Monocyte proliferation/migration	...
Placental growth factor	EC proliferation	2.93
Plasminogen activator	Degrading matrix molecules	...
Platelet-derived growth factor	SMC proliferation and migration	...
Stem cell–derived factor	Progenitor cell homing	...
Transforming growth factor-β	Vessel maturation, EC proliferation	2.11
Tumor necrosis factor-α	Degrade matrix molecules, EC proliferation	1.69
VEGF-A	EC proliferation, migration, tube formation	2.47
VEGF-B	EC proliferation, migration, tube formation	...

MCP-1 indicates monocyte chemoattractant protein-1; M-CSF, macrophage-specific colony-stimulating factor; VEGF, vascular endothelial growth factor; EC, endothelial cell; and SMC, smooth muscle cell.

suggested a close linear relationship between recovery of perfusion as assessed by LDPI, and positive remodeling of adductor collateral vessels.[14]

In Vivo Assessment of Limb Function

Functional assessment of the ischemic limb was performed using a modification of a clinical standard score.[15] A semiquantitative assessment of ambulatory impairment of the ischemic limb was performed serially (0=flexing the toes to resist gentle traction on the tail, 1=plantar flexion, 2=no dragging but no plantar flexion, 3=dragging of foot). A blinded observer assigned all scores.

Histological Assessment of Collateral Morphology

After completing blood flow assessment, sections of adductor muscles were stained with van Gieson's solution. Only arteries, identified by the presence of a continuous internal elastic laminae and muscle spindles, and with a mathematically derived area >300 μm^2, were counted. Total cross sectional area was calculated using Image-Pro software, with the smallest internal luminal distance measured as the radius.

Data and Statistical Analysis

Expression analysis data were verified by performing experiments in duplicate. All ELISA, immunoblotting, and cell studies were performed at least in triplicate. All results are presented as mean±SEM. Statistical significance was evaluated using an unpaired student *t* test, or ANOVA where indicated. A value of $P<0.05$ was considered significant.

Results

Gene Array of Human MSCs

MSCs expressed genes for a wide array of arteriogenic cytokines (Table). The expression of FGF-2, FGF-7, interleukin-1 and interleukin-6, placental growth factor, TGF-β, TNF-α, and VEGF-A were all augmented by exposure to hypoxic stress.

Cytokine Release From MSCs

To complement gene expression patterns, we analyzed the cytokine content of $hMSC^{CM}$ using ELISA (Figure 1). Baseline and hypoxic augmentation of VEGF-A secretion (375 pg/mg protein in normoxia versus 698 pg/mg in hypoxia; $P<0.01$) was confirmed with a similar pattern also seen for FGF-2 (2320 pg/mg versus 3970 pg/mg; $P<0.05$), and interleukin-6 (3885 pg/mg versus 7665 pg/mg; $P<0.01$), reflecting similar changes in gene expression. Although placental growth factor gene expression was augmented 3-fold by hypoxic stress, secretion of the cytokine was not significantly altered (119 pg/mg versus 164 pg; P=NS). Monocyte chemoattractant protein-1 gene expression was unchanged after exposure to hypoxic stress and a similar pattern of secretion was also seen (150 pg/mg versus 70 pg/mg; P=NS). Immunoblotting of the MSC^{CM} for angiopoetin-1, PDGF, metalloproteinase-9, and plasminogen activator also demonstrated similar cytokine release profiles to the expression profiles (Figure 2). ECs released minimal cytokines under baseline conditions and in response to hypoxia.

$hMSC^{CM}$ Effect on Endothelial Cell Proliferation and Migration

To examine whether $hMSC^{CM}$ exhibited biological effects relevant to collateral remodeling, a series of EC proliferation assays were performed. $hMSC^{CM}$ significantly enhanced EC

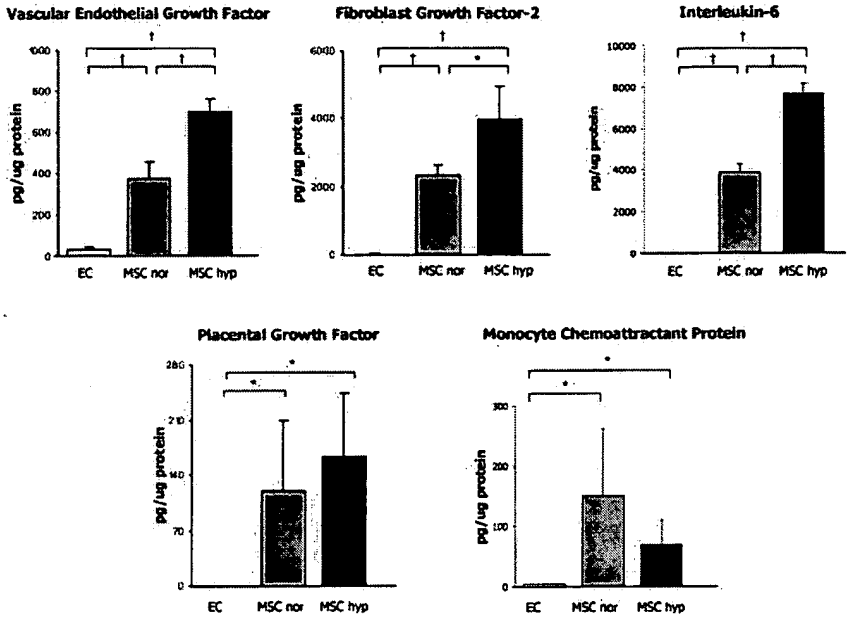

Figure 1. Cytokines released by MSCs in culture. In vitro release of VEGF, bFGF, IL-6, PlGF, and MCP-1 from marrow stromal cells as assessed by ELISA analysis of hMSC^{CM}. Data displayed from control ECs, hMSCs in normoxia, and hMSCs under hypoxic stress. *P<0.05, †P<0.01.

proliferation over control and was comparable to growth seen with EGM-2 (Figure 3A). The proliferative effect of hMSC^{CM} was completely abolished by boiling, suggesting these effects were due to a specific receptor/ligand interaction. As expected, the mitogenic effect of recombinant VEGF was inhibited by the addition of VEGF-blocking antibody (Figure 3A). In contrast to this, addition of the same antibody to hMSC^{CM} only partially attenuated EC proliferation (5.5-fold without antibody versus 3.6-fold with antibody; P<0.001; Figure 3B). Similarly, addition of an FGF-2 blocking antibody only partly attenuated proliferation (5.5-fold versus 4.6-fold, respectively; P<0.05, P<0.001 versus control media). The addition of both blocking antibodies significantly reduced the mitogenic effects, although the effect was still significant over control (P<0.001). There was no significant change in EC proliferation in response to hMSC^{CM} following the addition of anti-PDGF antibody. Additionally, reconstitution of DM-10 with recombinant VEGF to concentrations seen in the hMSC^{CM} dilutions failed to stimulate EC proliferation to the same extent as hMSC^{CM} (Figure 3C). The data strongly suggest that the mitogenic effects of hMSC^{CM} are due to multiple cytokines. Finally, a hMSC^{CM} dose-response relationship was also demonstrated (Figure 3C).

To further examine the biological effects of hMSC^{CM}, its effect on EC migration was studied. hMSC^{CM} induced a 5.5-fold increase in EC migration compared with control, although this did not achieve the fold increase observed with recombinant VEGF (Figures 4A and 4B). As with EC proliferation, boiling eliminated the chemoattractant properties of hMSC^{CM}.

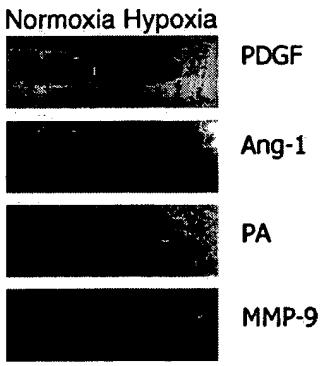

Figure 2. Immunoblotting of hMSC^{CM} levels of angiopoetin-1 (ang-1), platelet-derived growth factor (PDGF), metalloproteinase-9 (MMP-9), and plasminogen activator (PA).

hMSC^{CM} Effect on Smooth Muscle Cell Proliferation and Migration

hMSC^{CM} stimulated proliferation of SMCs in a dose-responsive manner, although this did not reach the effect seen with recombinant PDGF-β. As in ECs cultures, the SMC proliferative effect of hMSC^{CM} was abolished by boiling (Figure 5A). hMSC^{CM} also exerted a chemoattractant effect on SMCs, although the effect was only weak in comparison to PDGF-β and was inhibited by boiling (Figure 5B).

Hindlimb Blood Flow, Limb Recovery, and Collateral Morphology After mMSC^{CM} Injection

Having established that MSCs secrete many arteriogenic cytokines, and that the hMSC^{CM} exerts in vitro biological effects relevant to collateral remodeling, we proceeded to examine whether, as part of their therapeutic benefit, MSCs were able to contribute to collateral remodeling through paracrine mechanisms. To do this, we injected mMSC^{CM} directly into the adductor muscle (area of collateral remodeling) in a mouse model of hindlimb ischemia. In mice receiving control media, flow returned to ≈50% of the nonischemic limb by day 28. In contrast, in those mice receiving mMSC^{CM} there was a significant improvement in flow (Figure 6A) by day 3, which was maintained for the duration of the study (P<0.05 by ANOVA). Representative flow images are displayed in Figure 6B. In mice receiving MSC^{CM}, total arterial cross sectional area was significantly

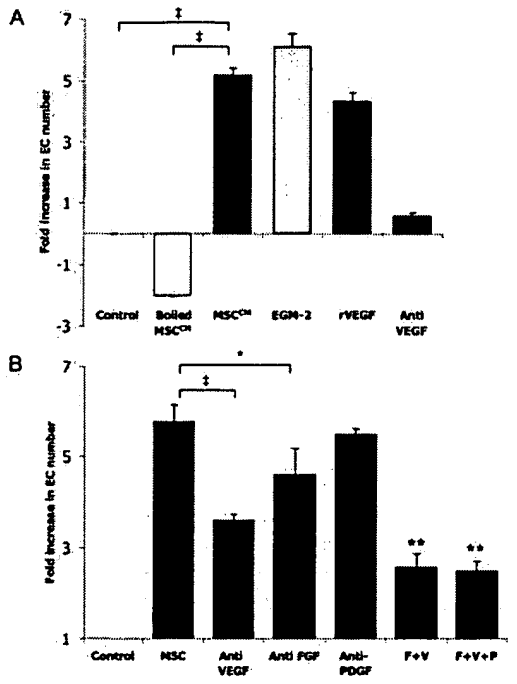

Figure 3. In vitro effects of hMSC^{CM} on EC proliferation and migration. A, Percent increase in the number of ECs over control media. EC proliferation with hMSC^{CM} approaches that seen with a commercial endothelial cell growth medium (EGM-2); this effect is abolished by boiling. Recombinant VEGF (rVEGF, 10 ng/mL) is used as a positive control and can be inhibited by the addition of blocking antibody. ‡$P<0.001$. B, Effects of blocking antibodies to VEGF (anti-VEGF, 10 μg/mL), FGF-2 (anti-FGF, 5 μg/mL), and PDGF (anti-PDGF, 5 μg/mL) on the EC mitogenic effects of hMSC^{CM}. Blocking VEGF or FGF-2 partially attenuated the response, although even the addition of both failed to totally abrogate the hMSC^{CM} effects (F+V, **$P<0.001$ vs control media). C, Graph demonstrating a dose-response curve between hMSC^{CM} dilutions and EC proliferation (filled squares). Reconstitution of control media with recombinant VEGF to the same concentrations as in the hMSC^{CM} dilutions failed to stimulate EC proliferation to the same degree (open squares). As before, addition of the same blocking VEGF antibody to the MSC^{CM} only partially attenuated proliferation (open circles).

increased in those mice compared with control (8380 μm^2 versus 4303 μm^2; $P<0.05$; Figure 6C).

The improved flow recovery was associated with improved hindlimb appearance and function. Mice receiving control media experienced severe ischemic damage resulting in a 60% incidence of autoamputation by day 28. However, mice receiving mMSC^{CM} displayed less ischemic damage with a 20% autoamputation rate. Similarly, in mice receiving mMSC^{CM}, limb function was significantly better than those mice receiving control media (ambulatory score 2.25 versus 1.25, respectively; $P<0.05$; Figure 6D).

Improved flow recovery also attenuated the calf muscle atrophy observed after femoral artery ligation. In control mice, muscle loss was significantly greater than in those mice receiving mMSC^{CM} (69% versus 41%, respectively; $P<0.05$; Figure 6E).

Discussion

Cells of the marrow stroma maintain hematopoietic stem cells and their progeny through a variety of molecular mechanisms including direct cell-to-cell interactions and, importantly, through local release of supportive cytokines.[16,17] Isolated reports have previously demonstrated release of VEGF and bFGF, but the present study is the first to our knowledge to definitively characterize the full spectrum of arteriogenic cytokines released by marrow-derived stromal cells. In addition, previous studies have documented marrow stromal cell secretion of hepatocyte growth factor,[18] insulin-like growth factor,[19] and MCP-2/MCP-3,[20] although mRNA for these cytokines was not found in the present study. Importantly, hypoxia also led to increases in the mRNA expression and secretion of several important cytokines such as VEGF and FGF-2 without adversely affecting release of any other cytokines. This is of relevance as the milieu into which cells are injected, such as ischemia versus nonischemia, is likely to have a major influence on their subsequent behavior.

The ability of bone marrow cells to secrete multiple arteriogenic cytokines has led to several studies demonstrating these cells enhance collateral flow, and the responsible mechanism has often been ascribed to these cells incorporating into the developing collaterals. However, the actual magnitude of incorporation of bone marrow–derived cells into vascular structures varies substantially among studies. Although some studies report over 50% of capillaries con-

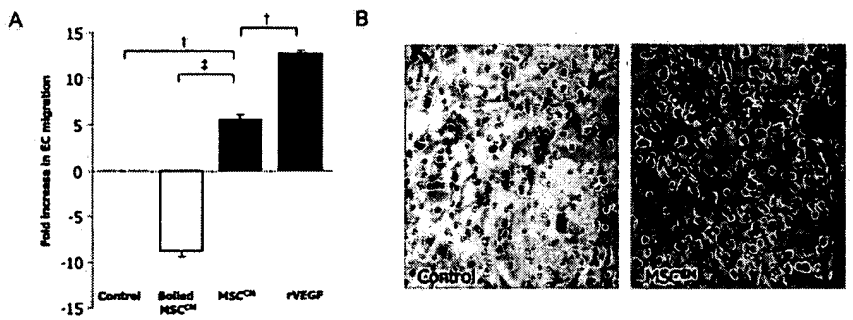

Figure 4. A, EC chemoattractant properties of hMSC^{CM}. There is a significant increase in EC migration seen with hMSC^{CM}, although it is less than that seen with recombinant VEGF (rVEGF, 10 ng/mL) control. †$P<0.01$, ‡$P<0.001$. B, Representative Transwell membranes stained with Hema-3, illustrating significant increase in EC migration compared with control.

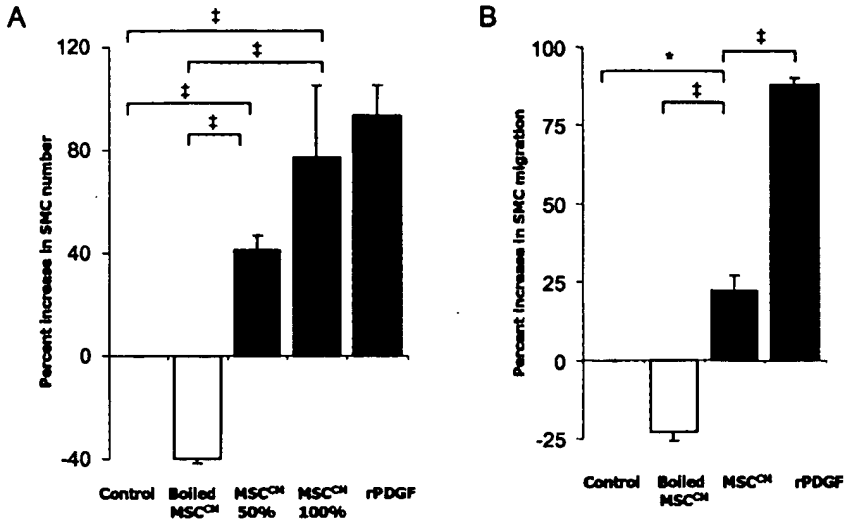

Figure 5. In vitro effects of hMSC^{CM} on SMC proliferation and migration. A, Percent increase in the number of SMCs over control media. Dose response is apparent as indicated by 50% and 100% dilutions of hMSC^{CM}, and this effect is abolished by boiling. Proliferation with recombinant PDGF (rPDGF, 10 ng/mL) as a positive control. ‡P<0.001. B, SMC chemoattractant properties of hMSC^{CM}. Mild increase in SMC migration seen with hMSC^{CM}, which is abolished by boiling. Chemoattractant effects seen do not reach that observed with recombinant PDGF control (rPDGF, 10 ng/mL). *P<0.05, ‡P<0.001.

taining transplanted cells, other studies have reported only occasional positive vessels despite noting impressive improvements in perfusion.[8–10] Taken together, these data suggest that other mechanisms apart from cell incorporation contribute to collateral remodeling observed after bone marrow–derived cell therapy in various models of ischemia.

The present study demonstrates that numerous arteriogenic cytokines are released by MSCs and, importantly, that injection of cells themselves is not required for therapeutic benefit, but that the release of such cytokines is sufficient to mediate arteriogenesis and enhance collateral flow. However, it is likely that complimentary mechanisms may contribute to the beneficial effects on blood vessel formation seen after cell therapy. Marrow stromal cells—also termed mesenchymal stem cells—have been demonstrated to differentiate into smooth muscle and endothelial cell lineages,[21–24] and thus may contribute cells directly to new or remodeling vessels. Nonetheless, the importance of the mechanism is still controversial.

Cytokines have not only individual effects, but one cytokine may potentiate (or inhibit) the effect of another. A synergistic relationship between VEGF and bFGF was reported in a rabbit ischemic hindlimb model, and placental growth factor appears to potentiate the effects of VEGF, both in in vitro and in vivo models.[25,26] Other studies have demonstrated synergism between PDGF and FGF-2 as well as between angiopoietin-1 and VEGF.[27,28] The present study also demonstrates this synergism. Blocking the effects of VEGF and bFGF in MSC^{CM} only partly attenuates the mitogenic effects of the MSC^{CM} on endothelial cells. Reconstitution of control media with recombinant VEGF to similar levels as that found in the MSC^{CM} stimulates endothelial cell proliferation, but not nearly to the extent as achieved with whole MSC^{CM}. Taken together, these data imply that multiple cytokines secreted by MSCs have additive or synergistic effects on cell proliferation, and as such MSC therapy may be more effective than single protein approaches in augmenting

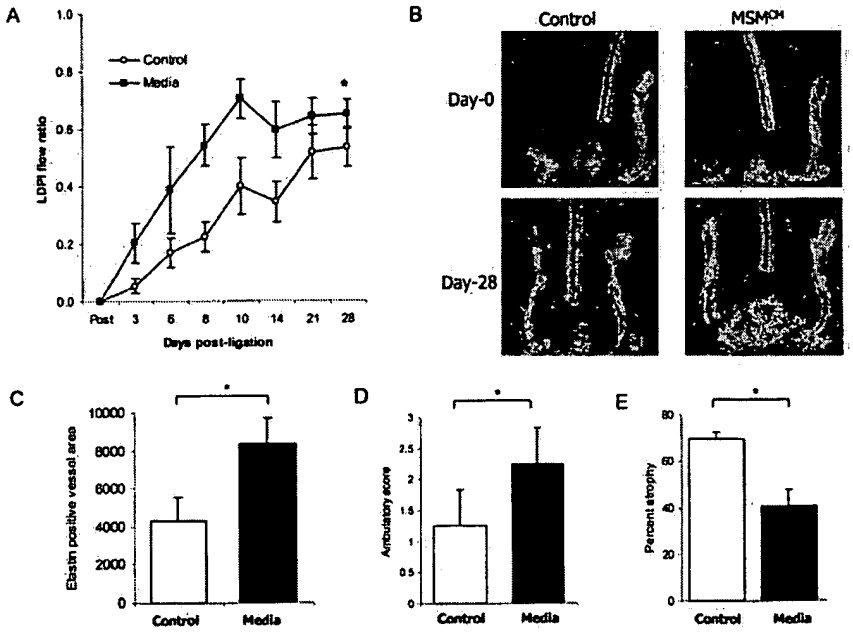

Figure 6. In vivo effects of mMSC^{CM}. A, Perfusion analysis after mMSC^{CM} treatment. LDPI expressed as a percentage of the normal limb. Flow recovery in mMSC^{CM}-treated animals was significantly better than that seen in animals treated with control media (*P<0.05 by ANOVA). B, Representative LDPI images of flow recovery in a mouse receiving mMSC^{CM} versus a mouse receiving control media. Red is highest velocity, green intermediate, and blue, lowest velocity. Postligation and day-28 images are displayed. C, Total cross-sectional area of elastin-positive vessels in the adductor muscle of mice treated with mMSC^{CM} versus control media (*P<0.05). D, MSC^{CM} injection led to a significant improvement of ambulatory function over control (*P<0.05). E, mMSC^{CM} injection significantly reduced calf atrophy versus control media (*P<0.05).

tissue perfusion. It is also interesting to speculate that MSC^{CM} could be used therapeutically rather than MSCs themselves, thus avoiding many practical issues regarding cell therapy.

Previous work examining the role of MSCs in angiogenesis demonstrated, using a Matrigel implantation model, that MSCs could augment capillary in-growth through paracrine mechanisms.[29] In that study, MSCs out to passage 14 were used, and their effects could be completely inhibited by addition of neutralizing anti-VEGF antibodies. These observations contrast with the present results. However, we have observed a gradual decrease over time in the release of PlGF and bFGF in MSC cultures (data not shown), whereas VEGF and MCP-1 levels remain relatively constant up to 4-weeks. Thus changes in the cytokine release profile over time may explain differences between this and previous studies. In the clinical setting, therefore, the timing of cell harvest may have important consequences for cell therapy in patients.

Given the importance of paracrine signaling in MSC/hematopoietic cell interactions, it is perhaps not surprising that MSC can augment collateral remodeling through paracrine mechanisms. However, previous studies have suggested this phenomenon may not be restricted to MSCs and that other bone marrow–derived cells may also influence blood flow recovery through release of arteriogenic cytokines. For example, bone marrow mononuclear cells contain mRNA for VEGF, bFGF, and angiopoietin-1, and after injection of BM mononuclear cells, local increases in VEGF protein have been observed.[30–32] Interestingly, injection of human-derived angioblasts into infarcted rat myocardium appeared to stimulate local host endothelial cells to proliferate, suggesting that these angioblasts may be a source of proangiogenic factors.[33] Endothelial progenitor cells in vitro also release several relevant cytokines, including VEGF and GM-CSF.[34] Thus, previous data in combination with the present study imply that bone marrow–derived progenitor cells can improve tissue ischemia in part through paracrine mechanisms. However, the exact degree to which this occurs is likely to vary from cell-to-cell and from milieu-to-milieu.

In summary, our data demonstrate that marrow-derived stromal cells secrete a broad spectrum of cytokines, which in vitro stimulate endothelial and smooth muscle cells to proliferate and migrate. These effects are dose-dependent and appear to be mediated by several cytokines. Furthermore, local injection of marrow stromal cell–derived conditioned media alone enhances collateral perfusion and remodeling in a murine model of hindlimb ischemia, reducing tissue atrophy and limb damage, and improving limb function, suggesting that paracrine signaling is an important mediator of bone marrow cell therapy in tissue ischemia.

Acknowledgments

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